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# Acute and Chronic Effects of Diflubenzuron (Dimilin) on Freshwater Fish and Invertebrates

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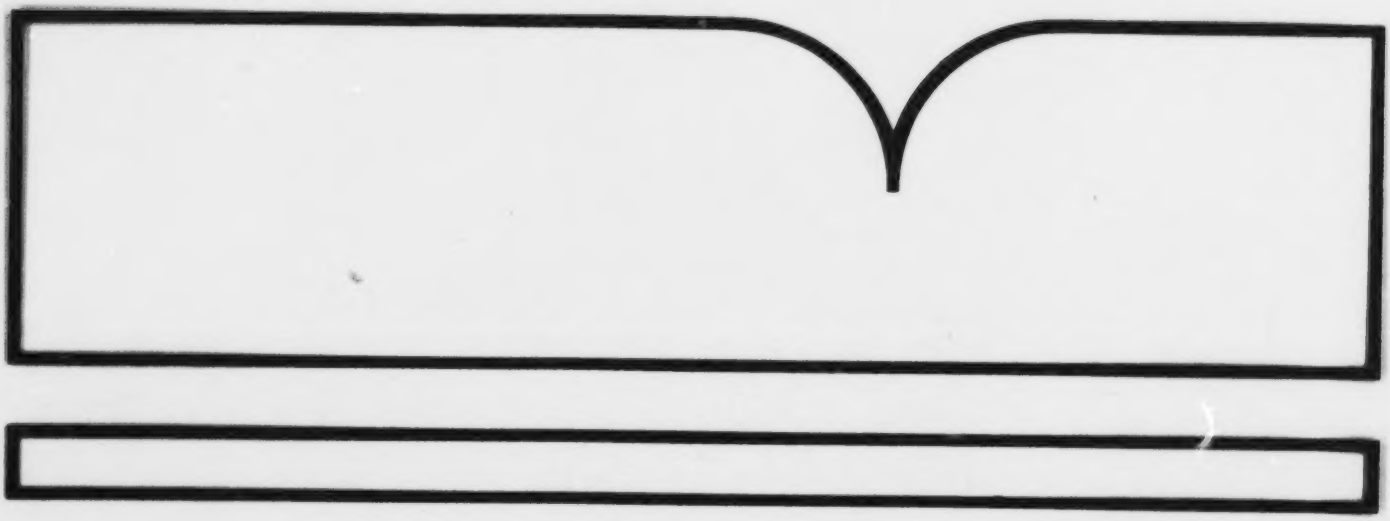

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# ACUTE AND CHRONIC EFFECTS OF DIFLUBENZURON (DIMILIN) ON FRESHWATER FISH AND INVERTEBRATES

ALAN V. NEBEKER,* PHILLIP MCKINNEY and MICHAEL A. CAIRNS

U.S. Environmental Protection Agency
Corvallis Environmental Research Laboratory
Corvallis, Oregon 97333

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**Abstract** — Two fish and seven invertebrate freshwater species were exposed to diflubenzuron (Dimilin®) in acute and chronic laboratory tests. No effects on newly hatched and juvenile fathead minnows or juvenile guppies were seen at or below 36 μg/L, the highest concentration tested. An early-life-stage test (30-d) with fathead minnows showed no effect at or below 36 μg/L. No effects on survival, growth or reproduction were observed with two snail species, *Juga plicifera* and *Physa* spp., at or below 36 μg/L. Adult emergence of the caddis fly *Clistoronia magnifica* was inhibited at 0.1 μg/L. *Daphnia magna* were killed at 2.0 μg/L. *Hyalella azteca* mortality was significant at 2.0 μg/L. Molting and survival of the midge *Tanytarsus dissimilis* were affected at 4.9 μg/L. Molting and survival of the midge *Cricotopus* spp. were affected at 4.9 μg/L, and adult emergence did not occur at 1.6 μg/L.

**Keywords** — Diflubenzuron (Dimilin®) Fish Invertebrates Chronic exposure effects

## INTRODUCTION

The insect growth regulator diflubenzuron (Dimilin®), a chitin synthesis inhibitor, is a recently developed pesticide that has been approved by the U.S. Environmental Protection Agency (EPA) for control of the gypsy moth and the boll weevil. It is being considered for use as a mosquito control agent, and is expected to be widely used in insect control [1,2]. It has been shown to be very effective in inhibiting the molting of target insects such as Lepidoptera, Diptera and Coleoptera [3]. It has also been shown to disrupt development and reproduction in nontarget aquatic insects and crustaceans important as fish food organisms [4].

Standard acute tests with freshwater fish and invertebrate species [5,6] are well established and provide useful information on the potential toxic action of many chemicals. Longer chronic and full-life-cycle tests are also becoming available to provide more precise data on harmful effects of toxicants in water. Laboratory microcosms and streams are being developed to determine if single-species tests can predict the impact of chemicals on complex, laboratory stream interactions and, ultimately, field situations. The purpose of this article is to present information obtained from single-species tests with Dimilin so that the data will be avail-

*To whom correspondence may be addressed.

able for comparison with results from laboratory stream-system tests at this laboratory using the same water and the same toxic material [2,7].

## MATERIALS AND METHODS

Nine species of freshwater fish and invertebrates (Table 1) were tested in accordance with guidelines established by the U.S. EPA [8], using methods published by the EPA [6], ASTM [5], Nebeker [9] and Nebeker et al. [10]. Tests with the insects were conducted so that at least one molt would occur during the test period and the effects of Dimilin on chitin synthesis and subsequent mortality could be evaluated. The two fish species, fathead minnow (*Pimephales promelas*) and guppy (*Poecilia reticulata*), were reared at the U.S. EPA Western Fish Toxicology Station (WFTS) Corvallis, Oregon. Caddis flies, *Clistoronia magnifica* (Limnephilidae), were obtained from N. H. Anderson, Oregon State University, and cultured at WFTS using the methods developed by Anderson [11]. *Daphnia magna* were reared from WFTS cultures. The gammarid crustacean *Hyalella azteca* was obtained from Oak Creek Laboratory, Oregon State University, and cultured at WFTS. The snails *Juga plicifera* (*Oxytrema silicula*) were collected from the Alsea River, Benton County, Oregon, and were held at WFTS for several months prior to testing; the snails *Physa* spp. were collected from WFTS ponds and held for several weeks prior to testing. The midges *Tanytarsus dissimilis* (*Paratanytarsus parthenogenica*) were obtained from the Environmental Research Laboratory at Duluth (U.S. EPA) and were cultured through several generations at WFTS prior to testing. Other midges, *Cricotopus* spp., were collected from fish tanks at WFTS within a few hours of testing. All animals were fed prior to testing, but were not fed during acute tests. Those animals tested for longer than 96 h were fed during testing. Fish and snails were fed Oregon Moist fish pellets (OMP). Newly hatched fathead minnows were fed newly hatched brine shrimp. *Daphnia* were fed dry trout food pellets blended with yeast and algae (*Selenastrum capricornutum*). Caddis flies were given quartz sand to use as case material, and were fed wheat, OMP and frozen brine shrimp. Gammarids were fed OMP, young brine shrimp and powdered alfalfa leaves. The midges were given algae

Table 1. Species and criteria for tests

| Animals | Parameter evaluated | Duration | Size or age of animals | Type of test |
|---|---|---|---|---|
| Fathead minnow (*Pimephales promelas*) | Survival; 30-d embryo–larval survival and growth | 96 h; 30 d | 8 wks old; egg to juvenile | Flow-through |
| Guppy (*Poecilia reticulata*) | Survival | 96 h | 4 wks old | Flow-through |
| Water flea (*Daphnia magna*) | Survival | 48 h; 6 d | < 24 h old | Static and Flow-through |
| Amphipod (*Hyalella azteca*) | Survival | 5 d | 2–4 mm length | Flow-through |
| Snail (*Juga plicifera*) | Survival | 7 d | 3–6 mm shell length | Flow-through |
| Snail (*Physa* spp.) | Survival, growth and reproduction | 3 wks | 12–20 mm shell length | Flow-through |
| Caddis fly (*Clistoronia magnifica*) | Survival and adult emergence | 4 wks | 4th and 5th instars | Flow-through |
| Midge (*Tanytarsus dissimilis*) | Molting success | 5 d | 2nd instar | Static |
| Midge (*Cricotopus* spp.) | Molting success and adult emergence | 7 d | 4th instar | Static and Flow-through |

Tests conducted at 20°C, 25 mg/L (as $CaCO_3$) hardness, and 16-h light/8-h dark photoperiod.

(*S. capricornutum*), OMP and powdered alfalfa leaves for food and case-building material.

Water quality was routinely monitored to characterize the water supply and ensure its suitability [12]. Temperatures were monitored continuously and held at 20 ± 1°C. The photoperiod was set at 16-h light. Hardness (as $CaCO_3$), total alkalinity (as $CaCO_3$) and pH were 22 mg/L, 24 mg/L and 7.3, respectively.

A continuous-flow diluter capable of delivering 10 concentrations of toxicant, with an extra mixing box for the first chamber, was used for all flow-through tests and to prepare concentrations for static-renewal tests (Fig. 1). Dimilin was added to the incoming dilution water in the mixing box of the diluter with a syringe-injection pump set at a constant delivery rate. The syringe needle was inserted in a plastic Tygon line from a water pump that recirculated and more vigorously mixed the water in the mixing box. The water from the mixing box then flowed into the first chamber of the diluter and was serially diluted (0.5 dilution ratio) and delivered in glass lines to the test aquaria. For the static-renewal tests, water was siphoned from diluter aquaria into beakers so that flow-through and non-aerated static tests could be conducted with the same water and the same initial measured concentrations. Water for the 48-h tests with *Daphnia* was not renewed; water for the longer tests with *Daphnia* and the midges was renewed every other day.

The insecticide Dimilin [diflubenzuron: 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea] was used for testing. The highest nominal test concentration was 45 μg/L, determined to be the limit of solubility under the dosing and test conditions used [7]. Measured concentrations from the diluter for the high concentration averaged 36 ± 4.2 μg/L. Analysis of test concentrations of Dimilin in the water was by high-pressure liquid chromatography. The limit of sensitivity of this method was close to 0.1 μg/L. The carrier solvent for all tests was dimethyl formamide (DMF).

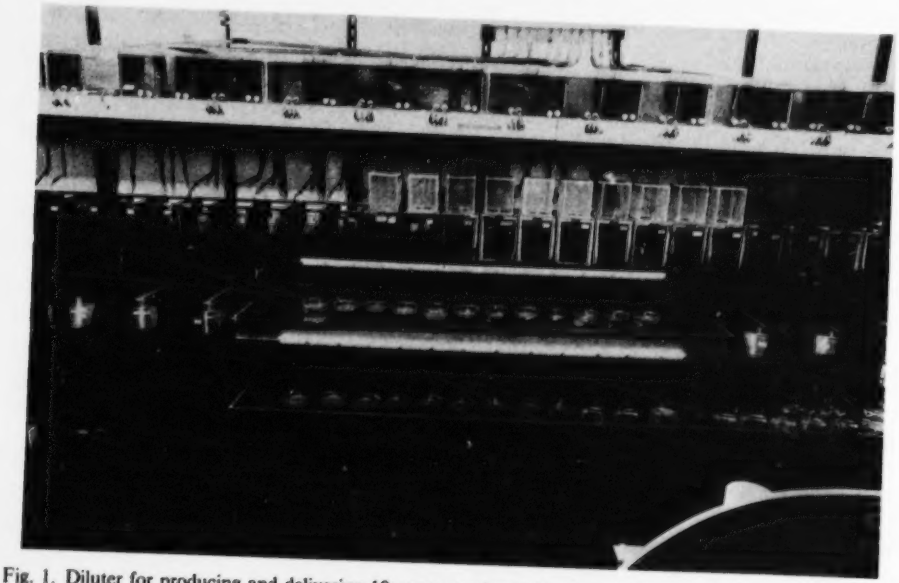

Fig. 1. Diluter for producing and delivering 10 concentrations of toxicant plus controls to replicated 10-liter aquaria (some with adult insect emergence cages), circulating artificial streams, 100-liter aquaria, and removable water baths (shown with 1-liter beakers for static tests with air lines for aeration). Freshwater and toxicant-delivery head boxes are behind fluorescent lights at the top of the photograph.

Time to death, molting success and adult insect emergence were determined, and the data were analyzed using analysis of variance and Dunnett's multiple comparison test [13] to determine significant differences from controls.

## RESULTS

No significant mortality ($p \geq 0.05$) was observed at or below 36 ± 4.2 μg/L during acute (96-h) or chronic tests with fathead minnows, or in the one acute test with guppies. One embryo–larval (early life stage) 30-d test was conducted with fathead minnow eggs and young fish; no significant effects on egg hatch, survival or growth were observed at 36 μg/L. A possible effect of DMF was observed when the freshwater control fish showed a slightly higher growth rate than did fish in the solvent control and Dimilin concentrations, but the difference was not significant. An increase in bacterial growth with DMF as the nutrient substrate was observed in the diluter and test chambers, necessitating more frequent cleaning of solvent control tanks and the higher-concentration tanks.

No effects were observed at or below 36 μg/L for either of the two snail species in tests of up to 3 weeks' duration. Good production of egg masses was observed for *Physa* spp. during the tests at 36 μg/L, and shell length doubled during the test period. *Juga* controls and test animals survived, but did not grow as well as did *Physa* spp., and they did not reproduce in the laboratory.

The adult emergence test with the caddis fly *Clistoronia magnifica* lasted for 4 weeks, starting with 5th-instar larvae and progressing through pupae development and adult emergence. Larvae died during the molt period between the 5th larval instar and the pupal stage at concentrations of 0.14 μg/L and greater. Three (30%) swimming pupae (pharate adults) formed and were active at 0.1 μg/L, but the adults never emerged and the swimming pupae died. No concentrations less than 0.1 μg/L were tested. Adult emergence with subsequent copulation occurred in the controls and carrier controls, indicating that Dimilin had a definite impact on the survival and adult emergence of this caddis fly species.

Several static and continuous-flow acute and adult emergence tests were completed with the chironomid midge *Cricotopus* spp. Dimilin prevented molting from 4th instar to pupae at and above 4.9 μg/L in continuous-flow tests, and adult emergence did not occur at 1.6 μg/L (Table 2). Tests with *Cricotopus* spp. under static conditions also showed significant mortality at 4.9 μg/L (Table 3).

The chironomid midge *Tanytarsus dissimilis* was tested to evaluate the effect on larval molting between the 2nd and 3rd larval instars. Significant effects ($p < 0.05$) were observed at and above 4.9 μg/L. Active 3rd-instar larvae developed at and below 2.0 μg/L (Table 4).

Tests were completed with the gammarid *Hyalella azteca*, but not all animals molted. Tests were conducted for 5 d using small juveniles (2–4 mm in length). Significant impact ($p < 0.05$) on survival was observed at and above the lowest concentration tested, 2.0 μg/L (Table 5).

Two tests were completed with *Daphnia magna*. In both the 2-d and the 6-d continuous-flow tests, Dimilin was toxic to *Daphnia magna* at a concentration of 2.0 μg/L, the lowest concentration tested. It was apparent that the safe level for Daphnia is less than 2 μg/L (Table 6).

## DISCUSSION

Dimilin does not give a typical dose–response curve over time such as is seen with most toxicants (e.g., copper or parathion); mortality occurs when the molting process is blocked. Because of this mode of action, the 24-h or 96-h $LC_{50}$ is of little value unless young animals that molt within the test period are used. The effective dose is related to molting and not to duration of exposure to Dimilin.

Table 2. Survival, molting success, and adult emergence of the midge *Cricotopus* spp. exposed to Dimilin as 4th-instar larvae (flow-through test)

| Mean measured concentration (μg/L) | No. of animals (4th instar) | Successful molt to pupae after 96-h exposure | | Percent successful adult emergence |
|---|---|---|---|---|
| | | No. of animals | % | |
| Control | 10 | 8 | 80 | 80 |
| Carrier control | 10 | 5 | 50 | 80 |
| Dimilin | | | | |
| 36.0 | 10 | 0 | 0[a] | 0 |
| 18.9 | 10 | 0 | 0[a] | 0 |
| 8.3 | 10 | 0 | 0[a] | 0 |
| 4.9 | 10 | 0 | 0[a] | 0 |
| 2.0 | 10 | 3 | 30 | 0 |
| 1.6 | 10 | 7 | 70 | 0 |

[a]Significantly different from controls ($p \leq 0.05$).

Most freshwater studies with Dimilin have used either static conditions in the laboratory or were tests in the field, where exposure concentrations were obtained by single or multiple application at weekly or monthly intervals. Few water samples were taken during the studies to verify actual Dimilin concentrations [14]. Continuous-flow tests in the laboratory, where the animals are exposed to constantly replenished toxicant levels for many days, have not been conducted as extensively with freshwater organisms, as they have been with some marine species [15]. Continuous-flow diluter tests in the laboratory were chosen for our studies so that constant concentrations of Dimilin in the test water could be accurately maintained, not so that field conditions could be simulated. Field application of Dimilin, in the range of 0.02 to 0.04 lb/acre (0.1–0.2 mg/L) [16], has demonstrated an initial decrease in concentration soon after contact with the water, but the concentration levels have been shown to remain fairly constant for several days to several weeks, usually depending on the method of application and the concentration in the sediments. If sand-grain application is used (sand coated with Dimilin applied to bodies of water) the concentration at the sediment–water interface is high and the concentration in the water above the sediment remains elevated due to the continued release of Dimilin from the coated sand. If wettable powder (25% active ingredient) is used, the initial concentration at the water surface is high but declines fairly rapidly as the Dimilin disperses into the water column. Dilution of

Table 3. Survival and adult emergence of the midge *Cricotopus* spp. exposed to Dimilin as 4th-instar larvae (static test)

| Mean measured concentration (μg/L) | No. of animals | Successful adult emergence after 7-d exposure | |
|---|---|---|---|
| | | No. of animals | % |
| Control | 20 | 18 | 90 |
| Carrier control | 20 | 11 | 55 |
| Dimilin | | | |
| 36.0 | 20 | 0 | 0[a] |
| 18.9 | 20 | 0 | 0[a] |
| 4.9 | 20 | 1 | 5[a] |
| 1.6 | 20 | 11 | 55 |
| 0.4 | 20 | 11 | 55 |
| 0.1 | 20 | 13 | 65 |

[a]Significantly different from controls ($p \leq 0.05$).

Table 4. Survival and molting success of the midge *Tanytarsus dissimilis* exposed to Dimilin as 2nd-instar larvae

| Mean measured concentration (μg/L) | No. of animals (2nd instar) | Successful molt to 3rd instar after 5-d exposure | |
|---|---|---|---|
| | | No. of animals | % |
| Control | 10 | 8 | 80 |
| Carrier control | 10 | 10 | 100 |
| Dimilin | | | |
| 36.0 | 10 | 0 | 0[a] |
| 18.9 | 10 | 0 | 0[a] |
| 8.3 | 10 | 0 | 0[a] |
| 4.9 | 10 | 3 | 30[a] |
| 2.0 | 10 | 6 | 60 |
| 1.6 | 10 | 10 | 100 |
| 0.7 | 10 | 10 | 100 |

[a]Significantly different from controls ($p \leq 0.05$).

the treated water body by incoming water, or currents in ponds, can cause an apparent concentration decrease in Dimilin when, in fact, it does not appear to chemically degrade very rapidly [17]. Nominal concentrations (0.5 to 10 μg/L) applied to California lakes are in the same range as those found to affect animals in this study, indicating that serious damage may be resulting from field applications. Questions remain as to whether Dimilin degrades or becomes bound to sediment or particles and therefore not readily available to aquatic animals.

Results from our laboratory study using continuous-flow conditions and stable Dimilin concentrations were somewhat predictable on the basis of the available literature. The fish were not expected to be harmed, but we were not sure what would happen during the 30-d fathead minnow early-life-stage test. None of the fish, including the eggs and newly hatched fatheads, were harmed at any of the test concentrations. The snails were not affected by Dimilin; both species did well, with *Physa* spp. growing and reproducing in all concentrations. One flow-through laboratory study by Julin and Sanders [16] was similar to ours, using three species of nontarget aquatic invertebrates and four species of fish. They obtained a 48-h $EC_{50}$ of 15 μg/L with *Daphnia magna* and a 48-h $EC_{50}$ of 560 μg/L for the midge *Chironomus plumosus*. However, results from their tests did not reflect the unique mode of action of Dimilin, due to the short exposure time and the apparent lack of molting during the exposure period.

The longer tests conducted in the present study, designed to include larval molts and adult emergence, give a better indication of the effectiveness of Dimilin in killing insect and crustacean species at very low concen-

Table 5. Survival of the scud, *Hyalella azteca* exposed to Dimilin

| Mean measured concentration (μg/L) | No. of animals | Survival after 96-h exposure | |
|---|---|---|---|
| | | No. of animals | % |
| Control | 20 | 17 | 85 |
| Carrier control | 20 | 20 | 100 |
| Dimilin | | | |
| 36.0 | 20 | 6 | 30[a] |
| 18.9 | 20 | 3 | 15[a] |
| 8.3 | 20 | 7 | 35[a] |
| 4.9 | 20 | 8 | 40[a] |
| 2.0 | 20 | 12 | 60[a] |

[a]Significantly different from controls ($p \leq 0.05$).

Table 6. Concentration of Dimilin causing significant mortality of test animals

| Animals | Concentration (μg/L) |
|---|---|
| Fathead minnow (*Pimephales promelas*) | >36 |
| Guppy (*Poecilia reticulata*) | >36 |
| Water flea (*Daphnia magna*) | 2.0 |
| Amphipod (*Hyalella azteca*) | 2.0 |
| Snail (*Juga plicifera*) | >36 |
| Snail (*Physa* spp.) | >36 |
| Caddis fly (*Clistoronia magnifica*) | 0.1 |
| Midge (*Tanytarsus dissimilis*) | 4.9 |
| Midge (*Cricotopus* spp.) | 1.6 |

trations. The month-long adult emergence study with the caddis fly *Clistoronia magnifica* shows that concentrations as low as 0.1 μg/L can prevent completion of the life cycle. As has been shown by field studies, and verified in our laboratory study, *Daphnia magna* is also very susceptible to Dimilin, with death resulting at levels of 2.0 μg/L. Larval development and adult emergence of the midge *Cricotopus* spp. were blocked by 4.9 and 1.6 μg/L concentrations respectively, in continuous-flow tests; larval development was impaired at 4.9 μg/L in static tests, indicating that low concentrations of Dimilin are toxic to this species also. The midge *Tanytarsus dissimilis* had a sensitivity similar to that of *Cricotopus* spp., with death resulting at 4.9 μg/L but successful molting occurring at and below 2.0 μg/L. The gammarid crustacean *Hyalella azteca* had a similar sensitivity as well, with significant mortality occurring at and above 2.0 μg/L Dimilin. The insect and crustacean species were very sensitive to Dimilin, with death resulting at concentrations lower than those normally applied under field conditions. Significant effort will be required for the protection of these organisms as the use of Dimilin increases in the future.

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